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ARTEMIA HEMOGLOBINS: GENETIC VARIATION IN PARTHENOGENETIC AND ZYGOGENETIC POPULATIONS

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Brine shrimps are distributed throughout the world in inland salt lakes, coastal lagoons, and salterns in which salt is commercially produced by evaporation of sea water. The many populations have often been designated by one binomen, *Artemia salina* (Linnaeus) Leach. However, six groups of shrimp populations are reproductively isolated from one another (reviewed by Clark and Bowen, 1976). Four of these six sibling species, when reared in the laboratory under identical environmental conditions, can be identified by a combination of characterizations of esterases and NAD–dependent malate dehydrogenase isozymes (Bowen and Sterling, 1978).

When brine shrimps are reared in a hypersaline medium under a 100% oxygen atmosphere, no hemoglobin is detectable in the hemolymph. When shrimps are reared under air (20% oxygen), four hemoglobins appear: Hb–1, Hb–2, Hb–X, and Hb–3, in descending order of electrophoretic mobility on cellulose acetate (Bowen, Lebherz, Poon, Chow, and Grigliatti, 1969). Hb–X was seen so rarely that it was thought to be a genetic variant of one of the other three hemoglobins. It is now known that if shrimps are reared under a 7% oxygen atmosphere, all four hemoglobins are induced in almost every shrimp of the 30 populations studied in our laboratory. Hb–1 is an alpha homopolymer $(\alpha\alpha)_n$; Hb–2 is a heteropolymer $(\alpha\beta)_n$; Hb–X is a beta homopolymer $(\beta\beta)_n$. Because the beta locus has a higher threshold for induction than the alpha locus, Hb–X is present only when the oxygen partial pressure is greatly reduced (Bowen, Sterling, and Barkan, 1977). Hb–3 consists of 2n polypeptides which are neither alpha nor beta polypeptides (Sterling and Bowen, 1977). The value of n is estimated by means of two paradigms in the discussion section of this paper. The four proteins are coded by a minimum of three structural genes, none of which is closely linked to another (Sterling and Bowen, 1977).

The purpose of this paper is to characterize the prevalent hemolymph proteins in 30 populations which represent six sibling species of *Artemia*. Intrapopulation polymorphism in Hb–1, Hb–2, and Hb–3 will be contrasted in parthenogenetic and zygogenetic populations. The data suggest that five of the seven parthenogenetic populations are of monophyletic origin. The hemoglobins are used as markers to demonstrate that a rare male from a parthenogenetic population can transfer genes to a female from a zygogenetic population.

Materials and Methods

The six sibling species are: *Artemia franciscana* Kellogg, 1906; *A. tunisiana* Bowen and Sterling, 1978; *A. urmiana* Gunther, 1900; *A. monica* Verrill, 1869;

A. persimilis Piccinelli and Prosdocimi, 1968; and *A. parthenogenetica* Bowen and Sterling, 1978. The locations of 27 populations within these six sibling species are listed in Table I. Detailed descriptions of most collection sites are given by Clark and Bowen (1976). San Francisco shrimps were collected as cysts from saltern A18 of the Leslie Salt Company at the southernmost tip of San Francisco Bay.

Mono Lake shrimps were collected as adults from the lake and maintained in lake water at 23° C for less than a week before electrophoresis. All other shrimps were hatched from cysts, fed on a mixed yeast diet, and maintained in glass vials (21 mm I.D.) in 5 ml of "medium D" at 23° C in the dark. The culture method has been described in detail (Clark and Bowen, 1976). *A. persimilis* and *A. franciscana* shrimps reached maturity (vitellogenesis in females) in two weeks. *A. tunisiana, A. urmiana,* and *A. parthenogenetica* required more time to mature (3–5 weeks). The matings were single-pair matings observed for a two week period. Shrimps were transferred from one vial to the next in glass pipets. These pipets were placed in boiling water for 5 minutes or more before they were employed to pick up shrimps of different genotype.

All shrimps were maintained under air (20% oxygen) until maturity. The hemoglobin patterns shown in Figures 1–3 and the data summarized in Tables I–III were from adults maintained under standard conditions (mixed yeast diet and air above culture medium D with no additional ferric salts) with three exceptions. Mono Lake shrimps were maintained in lake water. Some Tallaboa and Greater Inagua adult shrimps were maintained under an atmosphere of 10% oxygen and 90% nitrogen for two weeks to induce a higher level of hemoglobin because some shrimps in these two Caribbean populations had a higher threshold for induction of Hb–1 and Hb–2 (see pattern #4 in Figure 1).

For each electrophoretic analysis, the hemolymph from a single shrimp was collected and stored at 4° C for less than two hours prior to the run. The "Tris"–glycinate discontinuous buffer system, polyacrylamide slab gel (0.75 × 160 × 105 mm), water-cooled cell, stains, and procedures have been described (Sterling and Bowen, 1977). No stacking gel was used above the 6% acrylamide resolving gel. Two or more internal standards (samples containing Hb-1 with relative mobility of 100) were run on each gel. The standards were usually obtained from San Francisco and Kutch shrimps. Electrophoresis continued until the Hb–1 ^{100} had travelled 90 mm through the gel. Gels were stained with Coomassie Blue, a general protein stain. Relative hemoglobin concentrations were estimated on an EC180 recording densitometer. Mobility values were estimated from the trailing edge of Hb–1 and Hb–2 and from the center of Hb–3 and slow protein bands.

Results

Hemoglobins: interpretation of electrophoretic patterns

Twenty-three zygogenetic populations were studied: the 20 listed in Table I (hemolymph studied by electrophoresis on slab gels) and three additional *A. franciscana* populations (studied only by electrophoresis on tube gels) from San Quintin, Baja California, Mexico; from Soap Lake, Washington, U.S.A.; and from James Island (Isla Santiago), in the Galapagos. In all of these 23

TABLE I

Relative mobility of the most frequent electromorph of each of three hemoglobins in 27 Artemia *populations (6% acrylamide slab gels). The prevalent electromorph in the San Francisco population was used as reference (with mobility of 100). Populations from adjacent localities are paired if the same pattern prevails in both. Each analysis was made on the hemolymph of one shrimp. Within each parthenogenetic population, all shrimps have both Hb-2 electromorphs. In the Hidalgo* persimilis *population, the faster and slower Hb-2 electromorphs are equally frequent and genotype frequencies follow the expected Hardy-Weinberg distribution.*

Population	Relative mobility of prevalent electromorph			Alpha locus polymorphism	Beta locus polymorphism	Number of analyses
	Hb-1	Hb-2	Hb-3			
Artemia franciscana						
1. Little Manitou Lake and Lake Chaplin, Saskatchewan, Canada	98	99	100	Yes	No	22
2. San Francisco Bay (SFR Pond) and Vallejo West Pond, Calif., U.S.A.	100	100	100	Yes	Yes	151
3. Moss Landing, Calif, U.S.A.	98	99	100	Yes	No	18
4. San Diego, Calif., U.S.A.	97	99	98	No	Yes	41
5. Great Salt Lake, Utah, U.S.A.	98	99	98	Yes	Yes	51
6. Quemado, New Mexico, U.S.A.	96	99	98	Yes	Yes	66
7. Kiatuthlanna Red and Green Ponds, New Mexico, U.S.A.	96	99	92	No	Yes	61
8. Pichilingue Island, Mexico	96	98	98	Yes	No	28
9. Tallaboa, Puerto Rico, West Indies	97	98	92	Yes	No	42
10. Greater Inagua Island, West Indies	97	98	98	Yes	No	9
11. Rockhampton, Queensland, Australia	98	99	100	Yes	No	15
Artemia tunisiana						
1. San Bartolomeo, Sardinia	97	97	87	No	No	10
2. Chott Ariana, Tunisia	97	97	87	Yes	No	19
Artemia urmiana						
1. Lake Urmia (Lake Rezaiyeh), Iran	100	104	98	Yes	Yes	17
Artemia monica						
1. Mono Lake, California	99	100	100	Yes	?	16
Artemia persimilis						
1. Carahue and Hidalgo, Argentina	96	97, 99	87	Yes	Yes	37
Artemia parthenogenetica						
1. Kutch and Madras, India	100	104	98	No	No	87
2. Odessa (Russia) and Sète (France)	100	103, 105	98	No	No	63
3. Yamaguchi, Japan	100	102, 106	98	No	No	26
4. Rottnest Island and Port Hedland, Australia	99	101	98	No	No	9

populations, there was a pronounced difference in male and female electrophoretic patterns. When shrimps were reared under air, the hemolymph of females contained more Hb–1 than Hb–2; the converse was true in males. Photographs which illustrate the sex differences in electrophoretic patterns have been published (Sterling and Bowen, 1977, pp. 427 and 435).

When shrimps were reared under "standard conditions" in culture medium D under air, the hemolymph contained an excess of alpha over beta polypeptides. Therefore, Hb–X, the beta homopolymer, was not detected. Hb–3 was always present in the hemolymph when shrimps were immature but often was not detected at 8 weeks of age or more. The amount of Hb–3 has been shown to decrease with age in a clone of shrimps of the same genotype, descended through five generations from a single parthenogenetic female (Bowen *et al.*, 1969). In the present study, Hb–3 mobilities showed slight variation in females of shrimps of the K3 clone descended through three generations from a single parthenogenetic Kutch female.

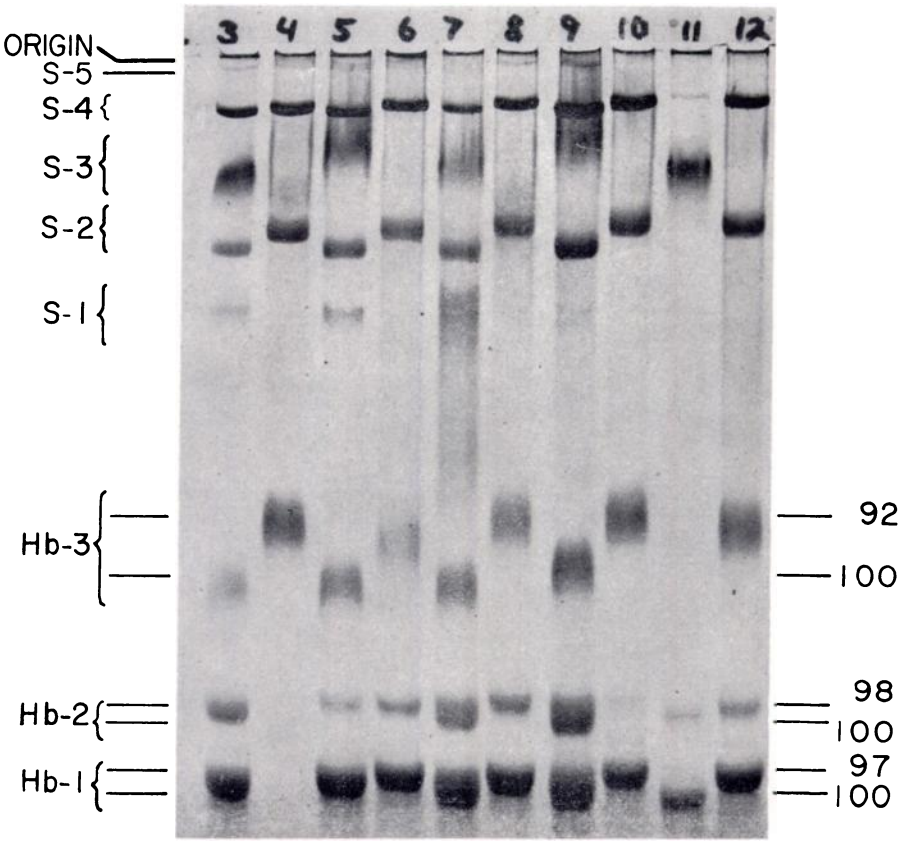

FIGURE 1. Hemolymph proteins from ten female shrimps (*Artemia franciscana*), showing three hemoglobins (Hb-1, Hb-2, and Hb-3) and five slow proteins (S-1 through S-5) which can be induced by low oxygen partial pressure in the 30 populations examined. Hb-X is not present because these shrimps were reared in culture medium under air. Hb-X appears in these populations when they are reared under an atmosphere of 7% oxygen. Shrimps #4, 6, 8, 10, and 12 are from Tallaboa; shrimps #3, 5, 7, 9, and 11 are from Vallejo. The common electromorphs in the Vallejo (and San Francisco) populations are assigned mobilities of 100. Shrimps #7 and #9 are heterozygous (α^{100}/α^{97}). Electrophoresis on 6% polyacrylamide slab gel, Tris-glycinate discontinuous buffer system, migration toward the anode (bottom of photograph).

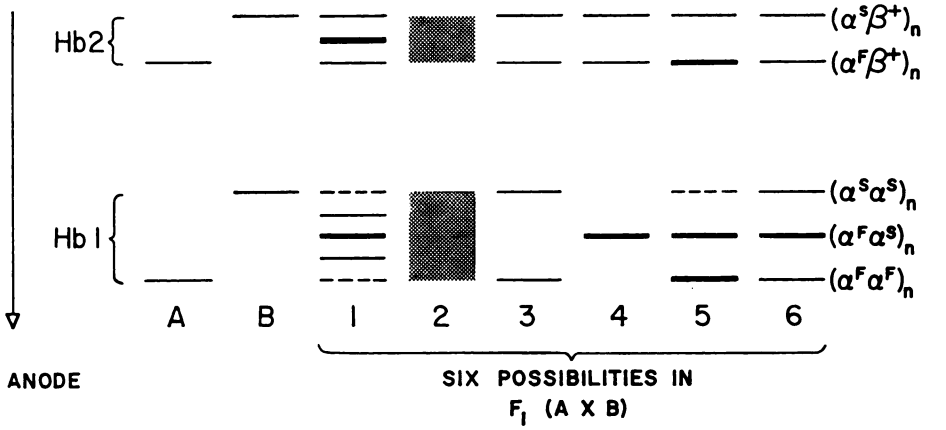

FIGURE 2. Predicted electrophoresis patterns of Hb-1 and Hb-2 in the hemolymph of F_1 progeny obtained by crossing homozygous shrimps A and B, genotypes $\alpha^F\alpha^F$; $\beta^+\beta^+$ and $\alpha^S\alpha^S$; $\beta^+\beta^+$, respectively. Possibility #6, a three-banded Hb-1 and two-banded Hb-2, was the most common pattern. It is the result of dissociation to dimers followed by reassociation of the dimers to form the native molecules. A few allelic combinations displayed pattern #5 (like #6 but with unequal amounts of the two alpha polypeptides) or #3 (further dissociation of $\alpha^F\alpha^S$ dimers to monomers before reassociation to form the native molecule). Possibility #2, a smear between the two parental extremes, was observed often in Hb-2 and more rarely in Hb-1. Patterns #1. (dissociation to tetramers) and #4 (greater affinity of unlike alpha polypeptides) were not documented in this study. An estimate of the value of n, the number of dimers in the native molecules, appears in the discussion section.

This suggests that this buffer system may not be optimum for this protein. Nonetheless, there are discrete mobility differences between populations (Figure 1 and Table I) and a three-banded Hb–3 is seen in the F_1 of certain racial crosses (Sterling and Bowen, 1977).

When two shrimps of different Hb–1 mobilities are mated, the F_1 progeny can be predicted to have six possible patterns (Figure 2). In most cases, pattern #6 was obtained: a three-banded Hb–1 and a two–banded Hb–2. The simplest interpretation is a rapid equilibrium of the native molecule with dimers. As the protein enters the gel, the dimers separate in the electric field, then reassociate to form native molecules composed of one kind of dimer (Bowen *et al.*, 1977). Possibilities #1 (dissociation to tetramers in Hb–1 and Hb–2) and #4 (preferential binding of unlike alpha polypeptides) were not documented in our study. Possibility #2 (a smear within the expected parental extremes of mobility) was often seen in Hb–2 but was only encountered in Hb–1 molecules containing the α^{99} and α^{98} alleles from San Francisco, Moss Landing, and Great Salt Lake. Possibility #3 (further dissociation of $\alpha^F\alpha^S$ dimers to monomers followed by reassociation to form the native molecules) was seen only in the combination of two San Francisco alleles (α^{100} and a rare α^{98}). Possibility #5 (like pattern #6 but with allelic products produced in unequal amounts) was seen when α^{92} from Quemado or α^{94} from Tallaboa was combined with α^{100} from San Francisco (or most other alleles). In both heterozygotes, the relative amounts of protein in the three bands of Hb–1 conformed to a binomial distribution, due to random assortment of the two alpha

polypeptides. These statements about the possibilities in Figure 2 are made after study of these alleles in classical genetics pedigrees (Sterling and Bowen, 1977). Examples of the most common pattern (#6 in Figure 2) have been illustrated in photographs (Bowen *et al.*, 1977; Sterling and Bowen, 1977).

Hemoglobins: genetic variation

The relative mobilities of the most frequent Hb–1, Hb–2, and Hb–3 electromorphs in 27 populations are listed in Table I. The reference in each case is the prevalent electromorph in the San Francisco population (with mobility of 100). Because there is so much interpopulation variation within *Artemia franciscana,* no one hemoglobin mobility can be used to identify with certainty any one of the six sibling species. The most striking characteristic is the low value of the relative mobility of Hb–3 in *A. persimilis* and *A. tunisiana.* However, only two populations of each species were examined and this relationship may not hold in future studies. The fact that Hb–1 has the same mobility in two populations is not meant to imply that the alpha alleles are identical. The Hb–1 in the Yamaguchi population is more sensitive to heat denaturation for 30 minutes at 50° C than is Hb–1 in the San Francisco population, although both have relative mobilities of 100 (procedure in Sterling and Bowen, 1977).

In Table I, the presence of alpha locus polymorphism was usually seen as a three–banded Hb–1, patterns #5 and 6, and more rarely as the alternative patterns #2 and 3 in Figure 2. The presence of beta polymorphism was seen as a one-banded Hb–1 with two–banded Hb–2 (Figure 3, samples B and F) or a three-banded Hb–1 combined with a four-banded Hb–2 (sample E).

Allelic frequencies at the alpha locus in six populations are listed in Table II. Three of the populations of *A. franciscana* include shrimps at two adjacent localities where allele frequencies are similar. No evidence of significant deviation from the

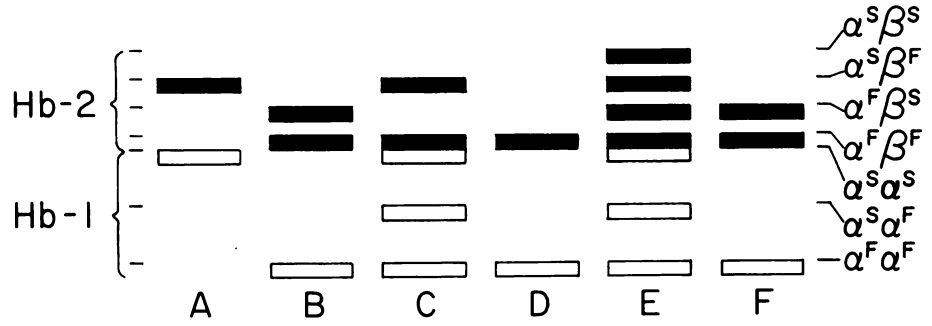

FIGURE 3. Tracing of photograph of electrophoretic patterns of six F_2 sibling shrimps from a parental cross of α^F/α^F; $\beta^F/\beta^F \times \alpha^S/\alpha^S$; β^S/β^S. Shrimps A and D are homozygous at both the alpha and beta loci and therefore each has only one species of Hb-1 and of Hb-2. Shrimps B, D, and F have the α^F/α^F genotype. Shrimps C and E have the α^F/α^S genotype and have a three-banded Hb-1. Shrimps A, C, and D are homozygous for the β^F allele. Shrimps B, E, and F are heterozygous at the beta locus. Shrimp E is heterozygous at both loci and the hemolymph pattern is a three-banded Hb-1 combined with a four-banded Hb-2. The genes are dervied from the San Francisco (SFR) and Quemado (QMD) populations. They are α_{SFR}^{100}, α_{QMD}^{09}, β_{QMD}^{F}, and β_{SFR}^{S}. Open bars: Hb-1; solid bars: Hb-2.

TABLE II

Hb-1 intrapopulation polymorphism in nine populations of A. franciscana *and in four populations of parthenogenetic shrimps. Data from three pairs of adjacent* franciscana *populations are pooled. Each analysis on hemolymph of one shrimp hatched from a cyst collected in the natural habitat.*

	Number of analyses	Frequencies of alpha alleles						
		100	99	98	97	96	92	Other
Artemia franciscana								
1. Lakes Chaplin and Little Manitou	22		0.03	0.97				
2. Kiatuthlanna ponds, New Mexico	61					1.00		
3. Quemado, New Mexico	66				0.02	0.87	0.11	
4. Pichilingue, Mexico	28				0.02	0.98		
5. SanDiego, California	41				1.00			
6. San Francisco and Vallejo	78	0.66						0.34*
Artemia parthenogenetica								
1. Kutch, Madras, Sète, Yamaguchi	176	1.00						

* San Francisco and Vallejo populations contained two or more alpha alleles which did not show the usual dissociation to dimers (pattern #6 in Figure 2).

Hardy–Weinberg distribution was observed in these shrimps hatched from cysts and reared in the laboratory. Heterozygosity is highest in San Francisco Bay shrimps. The exact value was not calculated because there are at least two alpha alleles which give patterns in heterozygotes which are difficult to interpret (dissociation patterns like those seen in Figure 2, possibilities #2 and #3). The other five *A. franciscana* populations cited in Table II are those which contain alpha and beta alleles which give unambiguous patterns (possibility #6 in Figure 2) and which have been studied in pedigrees (Sterling and Bowen, 1977). Hb–3 electromorph frequencies are not cited because resolution of this protein was not adequate in this buffer system. Hb–2 electromorph frequencies are not cited because this protein is a heteropolymer and both alpha and beta polypeptides show much variation in zygogenetic populations. Allelic frequencies at the beta locus are not given because these can be easily estimated only by determination of the relative mobilities of Hb–X, the beta homopolymer. Hb–X is consistently induced in every shrimp only when shrimps are reared for six weeks under 7% oxygen and this culture method was not used to maintain the shrimps described in Tables I through III.

Hemoglobins: genetic variation in threshold for induction

Both the alpha and beta loci are induced by low oxygen but the alpha locus has a lower threshold of induction. Thus, in San Francisco females reared by the standard method (under 20% oxygen), the amount of Hb–1 $(\alpha\alpha)_n$ exceeds the amount of Hb–2 $(\alpha\beta)_n$. When the same females are reared under 7% oxygen, the amount of Hb–2 exceeds that of Hb–1, and an additional protein, Hb–X or $(\beta\beta)_n$, appears in the hemolymph (Bowen *et al.*, 1977). In the present study, a lower threshold for induction (compared to the beta locus) was observed for the

alpha locus in Kutch, Madras, Sète, Yamaguchi, Urmia, Kiatuthlanna, Quemado, San Francisco, and Great Salt Lake shrimps.

Parthenogenetic females (Kutch, Madras, Yamaguchi, and Sète) contained more hemoglobin than did non-parthenogenetic females which in turn produced more hemoglobin than males. The sex difference was apparent in the Caribbean zygogenetic populations (Tallaboa and Greater Inagua) where the total amount of Hb–1 and Hb–2 was low. Within the San Francisco and Tallaboa populations there was variation in amount of Hb–1 and Hb–2 induced when shrimps were reared under the same standard conditions. A Tallaboa shrimp with undetectable Hb–1 and Hb–2 is shown in Figure 1, sample 4. We succeeded in selecting inbred lines with greater amounts and lesser amounts of Hb–1 than in average San Francisco shrimps (stocks derived from San Francisco and Tallaboa, respectively).

Slower migrating hemolymph proteins: evidence that S–2 and S–4 contain globin

Five "slow proteins," which migrate more slowly than the hemoglobins, were sometimes present in hemolymph. They are designated S-1 through S-5, in descending order of relative mobility on 6% acrylamide gels (Figure 1). They are not present in hemolymph when adult shrimps are reared with a 100% oxygen atmosphere above the culture medium. When oxygen is reduced to 10% (with 90% nitrogen), at least a trace of each of the five slow proteins can be detected in some shrimps in each population.

When reared under standard conditions (defined in Materials and Methods section), the female shrimps reproduce by birth of free-swimming nauplii. When ferric EDTA is added to the medium (30 mg/liter), a high proportion of the females produce encysted blastulae (cysts). The addition of ferric salts increases the concentration of S-1 twenty-fold in females.

Within the K3 clone, descended in the laboratory from one Kutch parthenogenetic female, the outline of S-3 is smeared and its relative mobility is variable, suggesting that this buffer system is not appropriate for this protein. S-5 is very concentrated in the hemolymph of *A. persimilis,* but often undetectable in other populations. It is seen near the top of the gel in sample #9 in Figure 1.

Proteins S-2 and S-4 were readily detectable in *A. monica* and in all populations of *A. franciscana* and *A. persimilis,* where their relative mobilities were correlated with those of Hb-3. This correlation is evident in Figure 1 and also in inbred stocks with uniform mobilities of Hb-1 and Hb-2 but varying mobilities of Hb-3 (Sterling and Bowen, 1977, photograph on p. 427). When three-banded Hb–3 patterns were seen in F_1 and backcross progeny of racial crosses, the S-2 bands of the heterozygotes showed a smear between the parental extremes of S-2 mobilities. This correlation of mobilities of S-2, S-4, and Hb-3, which is even more prominent on 5% gels, indicates that these two slow proteins and Hb-3 contain a common polypeptide.

When shrimps from San Francisco, Quemado, Yamaguchi, Carahue, and Tunis were reared under standard conditions (defined in Materials and Methods section of this paper), the hemoglobins were red, peroxidase positive (indicating the presence of heme) and were not stained with Oil Red O (which indicates the absence of lipid). The slow proteins were colorless, peroxidase negative, and did not stain with Oil Red O. However, when shrimps from the same populations were reared

under novel conditions (in a medium containing an additional 30 mg/liter of ferric EDTA, under 7% oxygen, and fed a diet containing algae), Hb-3, S-2, and S-3 were often stained by Oil Red O. When lipid was present, the proteins were often green with absorption maxima at 400 and 679 nm at pH 8.0. This suggests that carotene pigments were dissolved in the lipid. After cellulose acetate electrophoresis, the unstained bands of Hb-1, Hb-2, and Hb-3 were scanned for absorbance at 280 and 540 nm. The two values showed high correlation when shrimps were reared under standard conditions, suggesting a constant heme/protein ratio. The correlation was much lower when shrimps were fed a diet of algae or additional iron salts, suggesting variation in amount of heme bound to globin. The "Hb-3 band" was sometimes peroxidase negative and green before staining. This green protein was designated as "G-3" in an earlier study (Bowen *et al.*, 1969). When shrimps were reared under the same novel conditions, the five slow proteins were often both red before staining and peroxidase positive. Evidently, *Artemia* slow proteins and Hb-3 may be bound to varying amounts of heme and lipid *in vivo*.

Demonstration of interfertility of 13 Artemia franciscana *populations*

The evidence for reproductive isolation among the five zygogenetic sibling species listed in Table I has been reviewed by Clark and Bowen (1976). The evidence for interfertility of the populations grouped under *A. franciscana* will now be examined. In order to test for reproductive isolation, single pair matings were made between each unknown population and either San Francisco (SFR) shrimps or shrimps "cross fertile with San Francisco" (CRSFR). The CRSFR stock carries the recessive gene, ω, for white eyes and has been repeatedly backcrossed to SFR shrimps. The gene for white eyes is partially sex-linked; *i.e.*, it is on the homologous portion of the sex chromosomes (Bowen, 1965). This genetic marker was utilized in the racial crosses to exclude the possibility of gynogenesis (pseudogamy).

The data from cross-fertility tests of 12 populations are shown in Table III. In each cross and reciprocal cross, fertile F_1 and viable F_2 progeny were seen. When the white-eye gene was present, the expected F_2 segregation ratio (75% wild, 25% white) was seen. Omitted from the table are the control values for fertility in intrapopulation crosses. These ranged from a low of 6/29 (21%) in Quemado to 19/21 (90%) in Pichilingue shrimps. Because the San Francisco population is not reproductively isolated from the 12 populations in Table III, it is concluded that all are populations of *Artemia franciscana*.

Progeny of exceptional males from parthenogenetic populations

In the progeny of parthenogenetic females reared in our laboratory, we discovered four males. Two were found among the 1000 Madras females examined, one among 166 Sète females, and one among approximately 500 Yamaguchi females. Each male was mated to females from zygogenetic species. Three males (one each from Madras, Sète, and Yamaguchi) sired progeny which consisted of males and non-parthenogenetic females in approximately equal numbers. In the two crosses detailed below, the possibility of pseudogamy (gynogenesis) is excluded by the transfer of genetic markers from males to their progeny.

TABLE III

Hybridization of shrimps from 12 populations with shrimps from San Francisco (SFR) or an inbred line of white-eyed shrimps (w/w genotype) which are "cross-fertile with San Francisco" (CRSFR). In the parental cross, each wild-type shrimp hatched from a cyst collected in the natural habitat.

Parental cross Female × Male	Fertile pairs / total matings	Normal F_2 progeny obtained from each fertile parental pair	Normal F_2 segregation of gene (*w*) for white eyes
Great Salt Lake × CRSFR	19/34	Yes	Yes
CRSFR × Great Salt Lake, Utah	14/22	Yes	Yes
Greater Inagua × SFR	1/1	Yes	—
Greater Inagua × CRSFR	3/4	Yes	Yes
SFR × Greater Inagua Island	5/11	Yes	—
Kiatuthlanna Green × CRSFR	1/1	Yes	Yes
CRSFR × Kiatuthlanna Green Pond	4/6	Yes	Yes
Kiatuthlanna Red Pond × CRSFR	12/15	Yes	Yes
CRSFR × Kiatuthlanna Red Pond	4/4	Yes	Yes
Little Manitou × CRSFR	3/5	Yes	Yes
CRSFR × Little Manitou, Saskatchewan	8/10	Yes	Yes
Moss Landing × CRSRF	7/11	Yes	Yes
CRSFR × Moss Landing, California	9/10	Yes	Yes
Pichilingue × CRSFR	27/58	Yes	Yes
CRSFR × Pichilingue, Mexico	9/25	Yes	Yes
Quemado × CRSFR	6/11	Yes	Yes
CRSFR × Quemado	11/22	Yes	Yes
SFR × Quemado, New Mexico, U.S.A.	12/17	Yes	—
Rockhampton × SFR	3/8	Yes	—
Rockhampton × CRSFR	6/6	Yes	Yes
SFR × Rockhampton, Australia	2/2	Yes	—
San Diego × CRSFR	7/9	Yes	Yes
CRSFR × San Diego, California	9/13	Yes	Yes
San Quintin × CRSFR	3/4	Yes	Yes
CRSFR × San Quintin	2/2	Yes	Yes
SFR × San Qunitin, Mexico	9/13	Yes	—
Tallaboa × CRSFR	4/5	Yes	Yes
CRSFR × Tallaboa	2/2	Yes	Yes
SFR × Tallaboa, Puerto Rico	12/20	Yes	—

The Madras male (found by G.S.) was mated to the white-eyed CRSFR stock (*A. franciscana*) and sired 37 male and 38 female F_1 progeny, all of which had black eyes. Eleven F_1 females were isolated and none produced progeny over a five-week period. Another 17 were mated to their brothers and produced an F_2 generation with about equal numbers of males and females and the expected

segregation data for the white and sex loci. Forty-three of the F_2 females were isolated for a period of four weeks and none was parthenogenetic. We conclude that the gene (or genes) for parthenogenetic reproduction cannot be transmitted through the male.

The Yamaguchi male (found by S.T.B.) was mated to an *A. urmiana* female and sired two F_1 progeny. The F_1 male was mated to an *urmiana* female and produced seven F_2 offspring. The three F_2 females were not parthenogenetic. The exceptional Yamaguchi male and his F_1 daughter were tested for hemoglobins and two esterase isozymes. The hemolymph of the Yamaguchi male showed the hemoglobin pattern characteristic of Yamaguchi (the unique two bands of Hb-2 with mobilities of 1.02 and 1.06) but with greater amounts of Hb-2, relative to the amount of Hb-1, than is found in female Yamaguchi shrimps. His F_1 daughter had these two Hb-2 bands but the area between them was smeared, perhaps due to hybridization with the Hb-2 (with relative mobility of 104) typical of *urmiana*. The entire bodies of the exceptional male and his daughter were macerated and tested for the two "fast esterases" (Bowen and Sterling, 1978). The Est-2 was a single band of mobility intermediate between that of *A. urmiana* and the Yamaguchi population. The mobility of Est-1 could not be stated with certainty.

Although the transfer of genes from parthenogenetic populations to zygogenetic populations has been documented, this phenomenon must be extremely rare in the natural habitat due to the low incidence of male offspring of parthenogenetic females. Stefani (1964) reared Cagliari diploid parthenogenetic *Artemia* in the laboratory and reported the incidence of male offspring to be 0.004.

Discussion

The taxonomy of *Artemia* is in a state of flux because the morphological differences between sibling species vary with age, sex, and environmental influences. Many populations have been reported to consist of mixtures of sibling species (reviewed by Halfer-Cervini, Piccinelli, Prosdocimi, and Baratelli-Zambruni, 1968; Barigozzi, 1974). Early taxonomists assigned species names to shrimps at each locality and these names were often based on preserved specimens of different ages which were collected from salterns of different salinities. Therefore, we are aware that a few of the species names cited in this paper may be supplanted because some species might contain populations with name priority which have not yet been characterized by isozyme analysis nor by studies of reproductive isolation. Furthermore, taxonomic convention dictates that parthenogenetic clones bear the species name of the biparental population from which they are derived (Mayr, 1969). If the parthenogenetic clones can be shown (by inference from isozyme analysis) to be descended from, for example, *A urmiana,* then the name *A. parthenogenetica* would be supplanted by the more conventional *A. urmiana parthenogenetica.*

Taxonomic convention requires that the name *A. salina* be assigned to the shrimps observed in 1755 at the type locality (Lymington, England). Kuenen and Baas-Becking (1938) reviewed the contributions of Linnaeus, Lamarck, and Leach to the taxonomy of *Artemia* and printed a translation of the original description (by Schlosser in 1755) of the zygogenetic population at Lymington which was named by Linnaeus in 1758. Because two zygogenetic sibling species have been reported at one site in Europe (Halfer-Cervini *et al.,* 1968), it is uncertain which,

if any, of the species in the present study is identical to *A. salina Linnaeus*. It will be confusing to use the binomen *A. salina* to designate one of the European sibling species. Most biochemical studies have utilized California brine shrimps (*A. franciscana*) but have erroneously designated them as *A. salina* because the presence of sibling species was not recognized. We suggest that *A. salina* be discontinued as a name for populations in existence today.

The "slow proteins," which migrate more slowly than the hemoglobins, are a prominent feature in electrophoretic patterns of *Artemia* hemolymph proteins on 6% polyacrylamide gels (Figure 1). A discussion of these proteins is appropriate because two of the slow proteins, S-2 and S-4, contain a polypeptide in common with Hb-3. In starch gel electrophoresis of the hemolymph of *Parartemia zietziana*, Manwell (1978) found a colorless protein which migrated more slowly than the hemoglobins and which could bind heme *in vitro*. Perhaps the relationships of the four hemoglobins and five slow proteins of *Artemia* will someday be elucidated and they will be numbered consecutively. However, a new nomenclature would be premature at this time.

In order to estimate the value of n, the number of dimers in Hb-2 $(\alpha\beta)_n$, one must understand the structure of both the slow proteins and the hemoglobins. There is substantial agreement on the molecular weights of the native hemoglobin molecules. Hb-1, Hb-2, and Hb-3 have the same sedimentation coefficient (11.3S), which suggests a molecular weight of about 230,000 to 260,000 (Waring, Poon, and Bowen, 1970; Bowen, Moise, Waring, and Poon, 1976; Moens and Kondo, 1976, 1978; Manwell, 1978). The four hemoglobins and the genetic variants discovered to date have the same molecular size and conformation. Evidence for this is the fact that the relative distance between the proteins remains constant on cellulose acetate (no molecular sieving) as well as on 4, 5, 6, and 7.5% acrylamide gels (Bowen *et al.*, 1977). The molecular weight of the smallest hemoglobin subunit has been estimated from iron content and by relative electrophoretic mobility in SDS gels. Estimates of iron content of 0.29 and 0.299% (Bowen *et al.*, 1976; Moens and Kondo, 1978; respectively) have suggested a molecular weight per heme of 15,000–19,000 daltons. However, widely disparate conclusions were drawn from the SDS gel experiments. Moens and Kondo (1978) estimated that the smallest subunits obtained in SDS gels were of unequal size, 50,000 and 80,000 daltons, suggesting that each polypeptide was associated with more than one heme. They concluded that the native molecule was a dimer and that each of the two subunits (126,000 daltons) was composed of seven similar repeat units covalently linked to one another. Bowen *et al.* (1976) concluded that each polypeptide was the same size, about 15,000–19,000 daltons, and was associated with only one heme. The native molecule would consist of 12 polypeptides. It is now known that much free globin (hemoglobin apoprotein) is present at times in the hemolymph, suggesting that the previous estimate of 0.29% iron content in hemoglobins of saltern shrimps might have yielded too high an estimate of subunit molecular weight. The highest value for iron content of *Artemia* hemoglobin to be reported is 0.42% for one sample of Hb-1 (Moise and Bowen, unpublished). Because the number of polypeptides in the native molecule is a matter of controversy, the value of n may be unity or 6 or more, according to the paradigms of Moens and Kondo (1978) and of Bowen *et al.* (1977), respectively.

Because the ionic composition of the waters inhabited by *Artemia* varies more than that of any other aquatic metazoan, correlations were sought of allele frequencies with characteristics of habitat. In this study, shrimps originated from waters where the average salinity ranged from 54 to 210 g/liter (Mono Lake and Quemado, respectively). The ratio of chloride to carbonate ranged from 1.1 (Kiatuthlanna Green Pond and Mono Lake) to 810 (Lake Urmia). The ratio of sodium to potassium ranged from 8 (San Francisco) to 173 (Quemado). The ratio of chloride to sulfate ranged from 0.5 (Little Manitou) to 90 (Kiatuthlanna Red Pond). An interesting contrast was two inland lakes (Quemado and Kiatuthlanna Red Pond) only 85 km apart with chloride/carbonate ratios of 700 and 3 and chloride/sulfate ratios of 18 and 90, respectively. In these lakes, the most frequent electromorphs of Hb-1 and Hb-2 had identical mobilities. Although the prevailing Hb-3 was not the same in both populations, the most frequent Hb-3 mobility in one was the rare Hb-3 mobility in the other. Distinctive characteristics of electrophoretic pattern could not be associated with high total salinity, nor with high potassium, carbonate, or sulfate composition. It is possible that the electromorphs seen in different populations are the result of chance fixation of neutral alleles. The chemical composition of the waters inhabited by *Artemia* has been reviewed by Cole and Brown (1967).

A search for geographical patterns and latitudinal clines of alleles also yielded negative results. Although 2000 km apart, two parthenogenetic populations, Madras and Kutch, had identical mobilities for the three hemoglobins. Another two populations, San Francisco and Moss Landing, had different mobilities of the prevalent alleles although they were only 75 km apart.

Asexual reproduction is not necessarily associated with genetic uniformity. Saura, Lokki, Lankinen, and Suomalainen (1976) observed 20 isozyme loci in 127 populations of parthenogenetic tetraploid weevils of monophyletic lineage. The majority of loci were polymorphic. Genetic variation was evident within and between weevil populations.

In the present study, there was no intrapopulation variation in Hb-1 or Hb-2 phenotype among the parthenogenetic shrimps. This suggests that populations such as Kutch or Sète (57 shrimps from each population examined) are each derived from one clone which has recently invaded the habitat. It is not possible to comment on polymorphism in Hb-3 because there were slight variations in Hb-3 mobility within the progeny of a single Kutch female. The buffer system may be inappropriate for adequate resolution of this protein. The data in Table I suggest that Kutch and Madras are recently derived from the same clone, as are the Sète-Odessa pair and the Port Hedland-Rottnest pair. In Table I, if two Hb-2 mobilities are indicated in a parthenogenetic population (*e.g.*, Yamaguchi), every shrimp in that population had both bands of Hb-2. The Yamaguchi shrimps may all be heterozygous (B_1^{102}/β_1^{106}) or may show expression of a duplicate locus (homozyous genotype $\beta_1^{102}/\beta_1^{102}$; $\beta_2^{106}/\beta_2^{106}$).

Five of the seven parthenogenetic populations show striking similarities. Kutch, Madras, Sète, Odessa, and Yamaguchi have identical mobilities of Hb-1. All have Hb-2 species with relative mobilities greater than 100. They have identical patterns of esterases and NAD-dependent MDH isozymes which set them apart from the Port Hedland parthenogenetic population and ten zygogenetic populations

representing *A. tunisiana, A. urmiana,* and *A. franciscana* (Bowen and Sterling, 1978). This suggests that either these five parthenogenetic populations, all of which live in coastal salterns, have had the same alleles selected due to similarity of environment or all have descended with mutation from a monophyletic origin. The first explanation seems unlikely because zygogenetic *A. franciscana* shrimps from coastal salterns (San Francisco, Tallaboa, Moss Landing, Pichilingue, for example) have evolved different characteristic patterns of hemoglobin mobilities. The second explanation is more plausible: the five have originated from a single "transformation to parthenogenetic reproduction event" from a zygogenetic population. It will be interesting to see if this generalization holds as these strains are characterized further for biochemical traits.

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SUMMARY

1. Twelve populations of *Artemia* were found to be crossfertile with San Francisco shrimps, which indicates that they are *Artemia franciscana.*

2. Three hemoglobins (Hb-1, Hb-2, and Hb-3) were induced in shrimps from 30 populations. Although many populations had unique patterns (considering relative mobilities of all three hemoglobins) there was so much interpopulation variation among 14 populations of *A. franciscana* that no one protein was diagnostic of any one of the five sibling species of zygogenetic shrimps.

3. Intrapopulation polymorphism of Hb-1 and/or Hb-2 was seen in most zygogenetic populations but was not detected in parthenogenetic populations. This suggests that parthenogenetic populations such as Kutch or Sète (57 shrimps examined from each) are each descended from a single clone which has recently invaded the habitat. Five of the seven parthenogenetic populations have Hb-1 and Hb-3 electromorphs with the same relative mobilities, suggesting that they may have a monophyletic origin.

4. Two exceptional males (progeny of parthenogenetic females reared in the laboratory) were mated to females from zygogenetic populations. The progeny were equal numbers of males and non-parthenogenetic females. Transfer of genes from one exceptional male to an *A. urmiana* female was documented with three genetic markers.

5. The relationship of the hemoglobins and the "slow proteins" of the hemolymph is discussed.

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